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The orthotropic elastic properties of fibrolamellar bone tissue in juvenile white-tailed deer femora

John W. Barrera,[1] Adeline Le Cabec[2] and Meir M. Barak[1,3]

[1]*Department of Biology, Winthrop University, Rock Hill, SC, USA*
[2]*Department of Human Evolution, Max Planck Institute for Evolutionary Anthropology, Leipzig, Germany*
[3]*Department of Human Evolutionary Biology, Harvard University, Cambridge, MA, USA*

Abstract

Fibrolamellar bone is a transient primary bone tissue found in fast-growing juvenile mammals, several species of birds and large dinosaurs. Despite the fact that this bone tissue is prevalent in many species, the vast majority of bone structural and mechanical studies are focused on human osteonal bone tissue. Previous research revealed the orthotropic structure of fibrolamellar bone, but only a handful of experiments investigated its elastic properties, mostly in the axial direction. Here we have performed for the first time an extensive biomechanical study to determine the elastic properties of fibrolamellar bone in all three orthogonal directions. We have tested 30 fibrolamellar bone cubes ($2 \times 2 \times 2$ mm) from the femora of five juvenile white-tailed deer (*Odocoileus virginianus*) in compression. Each bone cube was compressed iteratively, within its elastic region, in the axial, transverse and radial directions, and bone stiffness (Young's modulus) was recorded. Next, the cubes were kept for 7 days at 4 °C and then compressed again to test whether bone stiffness had significantly deteriorated. Our results demonstrated that bone tissue in the deer femora has an orthotropic elastic behavior where the highest stiffness was in the axial direction followed by the transverse and the radial directions (21.6 ± 3.3, 17.6 ± 3.0 and 14.9 ± 1.9 Gpa, respectively). Our results also revealed a slight non-significant decrease in bone stiffness after 7 days. Finally, our sample size allowed us to establish that population variance was much bigger in the axial direction than the radial direction, potentially reflecting bone adaptation to the large diversity in loading activity between individuals in the loading direction (axial) compared with the normal (radial) direction. This study confirms that the mechanically well-studied human transverse-isotropic osteonal bone is just one possible functional adaptation of bone tissue and that other vertebrate species use an orthotropic bone tissue structure which is more suitable for their mechanical requirements.

Key words: deer; femur; fibrolamellar bone; plexiform bone; stiffness.

Introduction

Fibrolamellar bone (also called plexiform bone or laminar bone) is a primary bone tissue which is predominant in long bones of fast-growing juvenile mammals (Enlow & Brown, 1958; Currey, 2002; Locke, 2004; Mori et al. 2005; Hillier & Bell, 2007; Marín-Moratalla et al. 2011), at least several species of birds (Castanet et al. 2000; de Margerie et al. 2002, 2004), and large dinosaurs (Enlow & Brown, 1957; Horner et al. 2005; Stein & Prondvai, 2014). This bony structure is Nature's solution in cases when bones need to grow fast in diameter, faster than other bone tissue types (such as lamellar bone) can be laid on the periosteal surface (Currey, 2002). Usually, in large mammals, fibrolamellar bone is replaced by secondary Haversian bone tissue (i.e. remodeling) after the animal has matured (Mori et al. 2005).

The formation of fibrolamellar bone on the periosteal surface of bones occurs in several partially overlapping steps. Initially, a branched network of fine blood vessels develops on the inner surface of the periosteal fibrous layer; the primary direction of these blood vessels tends to be along the long axis of the bone and they serve as 'footing' for new bone to form (Currey, 1960). Soon after, a thin layer of porous and hypercalcified woven bone grows and encloses these blood vessels (Stover et al. 1992; Mori et al. 2003, 2007; Almany Magal et al. 2014); the thin woven layer extends outwards and is attached to the old sub-periosteal surface by thin bony struts ('bridges'). This is the quickest step in the formation of fibrolamellar bone and studies

Correspondence
Meir Max Barak, Department of Biology, Winthrop University, Rock Hill, SC 29733, USA. T: + 1 803 3232111; F: + 1 803 3233448; E: barakm@winthrop.edu

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indicate a growth rate of up to 80–100 μm a day (Castanet et al. 2000; de Margerie et al. 2002). This woven bone functions as scaffolding for the next step, in which slower growing parallel-fibered bone is deposited on both sides of the woven bone layer (Kerschnitzki et al. 2011; Almany Magal et al. 2014). Finally, lamellar bone is deposited concentrically in layers (lamellae) from the surface of the parallel-fibered bone toward the blood vessels, creating a sandwich-like structure of 'primary osteons' (Currey, 2002). This whole cascade of events occurs concomitantly; while parallel-fibered bone and then lamellar bone are being laid between the blood vessels and the thin woven bone scaffolding, a new thin layer of woven bone is already growing outwards, ever increasing the bone's diameter (a clear schematic view of the process can be found in Currey, 2002; figure 1.4; Currey, 2002). The end result is a brick-like configuration of bony units inhabiting primarily the periosteal region of the bone. Consequently, and contrary to Haversian bone, fibrolamellar bone tissue is an orthotropic structure rather than a transverse isotropic structure (Katz et al. 1984; Currey, 2002).

Fibrolamellar bone tissue is thought to be advantageous and superior to Haversian bone tissue in its fast growth rate and mechanical properties (specifically stiffness and strength) yet inferior in tissue toughness (fibrolamellar bone, a primary bone tissue, is devoid of cement lines which serve in arresting crack propagation). Although fibrolamellar bone tissue is by no means an infrequent structure in the animal kingdom, surprisingly relatively little research has been done on its mechanical properties, specifically its stiffness (as is true in many other cases – our anthropocentric approach leads us to concentrate and study mostly human-related features, osteonal bone in our case, even if they are only one of many possibilities). Starting around 40 years ago with the ground-breaking biomechanical bone experiments by Reilly et al. (1974) and Reilly & Burstein (1975), a handful of studies have investigated the stiffness properties of fibrolamellar bone (Table 1). These studies spanned various testing techniques (tension, compression, bending, and ultrasound) and frequently tested very few samples in only one orientation (usually axial), thus making it very difficult to compare studies and form definite conclusions. Furthermore, in some cases the authors either did not verify the tissue type of their samples and just assumed it was fibrolamellar bone (based on the animal's age), or even clearly stated that each sample contained a mix of 'fibrolamellar and Haversian bone'. Finally, the vast majority of these studies tested bovine bone, focusing only on one species and neglecting all other large mammal taxa.

Here we present for the first time an extensive study of fibrolamellar bone stiffness (Young's modulus) taken from the proximomedial diaphysis of five young white-tailed deer femora. Our current experiment introduces several advantages compared with previous studies and adds new information to current understanding of fibrolamellar bone biomechanics. First, our sample size is the largest to date – we tested 30 bone cubes harvested from five different femora (all but one of the previous studies used fewer than 20 samples and all studies used fewer than five individual animals). Secondly, by testing the bone samples in compression within their elastic region we were able to obtain all three orthogonal Young's moduli values for each sample (all previous studies tested each sample only in one orientation). In addition, loading the bone samples in compression tested the bone tissue in its physiological state (the femoral proximomedial diaphysis is subjected primarily to compression). Thirdly, as we were testing the samples within their elastic region, each sample was tested three times in each orientation, thus allowing us to obtain a much more precise value. Fourthly, we used a different species than that used so far (deer instead of bovine) and thus extended our understanding of bone biomechanics in less studied taxa.

Table 1 Summary of previous studies testing the stiffness of 'verified' solely fibrolamellar bone samples in the three orthogonal axes (axial, transverse and radial). Notice that all studies used just one species (bovine) and that except for one study (Van Buskirk & Ashman, 1981) each sample was measured in only one orientation.

Study	Animal	Bone	Testing technique	No. of samples (no. of individuals)	Loading direction	Stiffness (GPa)
Reilly et al. (1974)	Bovine	Femur (diaphysis)	Compression	10 (2)	Axial	30.3
	Bovine	Femur (diaphysis)	Tension	10 (2)	Axial	27.2
Reilly & Burstein (1975)	Bovine	Femur (diaphysis)	Tension	6 (1)	Axial	26.5
Van Buskirk & Ashman (1981)	Bovine	Femur (diaphysis)	Ultrasound	20 (1)	Axial	21.9
Lipson & Katz (1984)	Bovine	Femur (diaphysis)	Ultrasound	16 (4)	Axial	29.8
Martin & Boardman (1993)	Bovine	Tibia	3-point bending	35 (3)	Axial	21.0
Van Buskirk & Ashman (1981)	Bovine	Femur (diaphysis)	Ultrasound	20 (1)	Transverse	14.6
Lipson & Katz (1984)	Bovine	Femur (diaphysis)	Ultrasound	12 (4)	Transverse	21.4
Reilly & Burstein (1975)	Bovine	Femur (diaphysis)	Tension	25 (1)	Transverse/Radial	11.0
Van Buskirk & Ashman (1981)	Bovine	Femur (diaphysis)	Ultrasound	20 (1)	Radial	11.6
Lipson & Katz (1984)	Bovine	Femur (diaphysis)	Ultrasound	4 (4)	Radial	17.3

Finally, as our samples were of young animals and thus their percentage organic material (mostly collagen) was relatively high (and percentage mineral was relatively low), we retested the same samples after they were kept refrigerated for 7 days (4 °C) to evaluate whether bone stiffness deteriorated significantly.

In the current study we tested two hypotheses.

Working hypothesis (1). Fibrolamellar bone is an orthotropic structure and therefore it will demonstrate orthotropic stiffness behavior; more specifically, we expected that our samples would present the highest Young's modulus in the axial direction followed by the transverse and radial directions.

Working hypothesis (2). Since the relative organic mass in young animals is higher (lower mineralization) we postulated that after the samples were exposed to 4 °C for 7 days they would demonstrate a significant decrease in stiffness in all three orthogonal directions.

Material and methods

Bone sample preparation

Femora from five young white-tailed deer (*Odocoileus virginianus*) were obtained from a local processing factory right after the animals were captured and killed. The cause of death for all deer was not related to the musculoskeletal system and all femora were intact with no sign of any pathology. Sex and age were unknown but all femora had active growth plates, indicating these were juvenile individuals still in their growing phase. All soft tissue was removed and the bones were stored at −20 °C. Next, the femora were sectioned with a hand saw and 3-cm-thick segments were taken from the proximal diaphysis of each bone (Fig. 1A). Each proximal segment was cut into four cortical quadrants according to its anatomical orientations: medial, lateral, cranial and caudal. Five to eight cubic samples (2 × 2 × 2 mm, total of 30 samples) were prepared from the medial quadrant of each femur using a low-speed water-cooled diamond saw (Techcut 4 precision low speed saw, Allied Technologies). A sample size of 30 was chosen to exceed sample sizes of previous studies (see Table 1) and also to achieve an approximate normal distribution (stated by central limit theorem). The cubes were cut such that their faces were aligned with the anatomical axes of the bone: proximal-distal (axial), medial-lateral (radial) and cranial-caudal (transverse) orientations. The sample dimensions were measured to an accuracy of 0.01 mm and then stored for 30–90 days on water-saturated cloth at −20 °C.

Bone slide preparation

Concurrently to sample preparation, thin bone slices were cut proximal, distal and to the sides of the cube samples. These thin slices were used for a histological study of the tissue. Slices were inspected under a microscope (Nikon Eclipse E600) to confirm the fibrolamellar tissue structure of our cubical bone samples (Fig. 2).

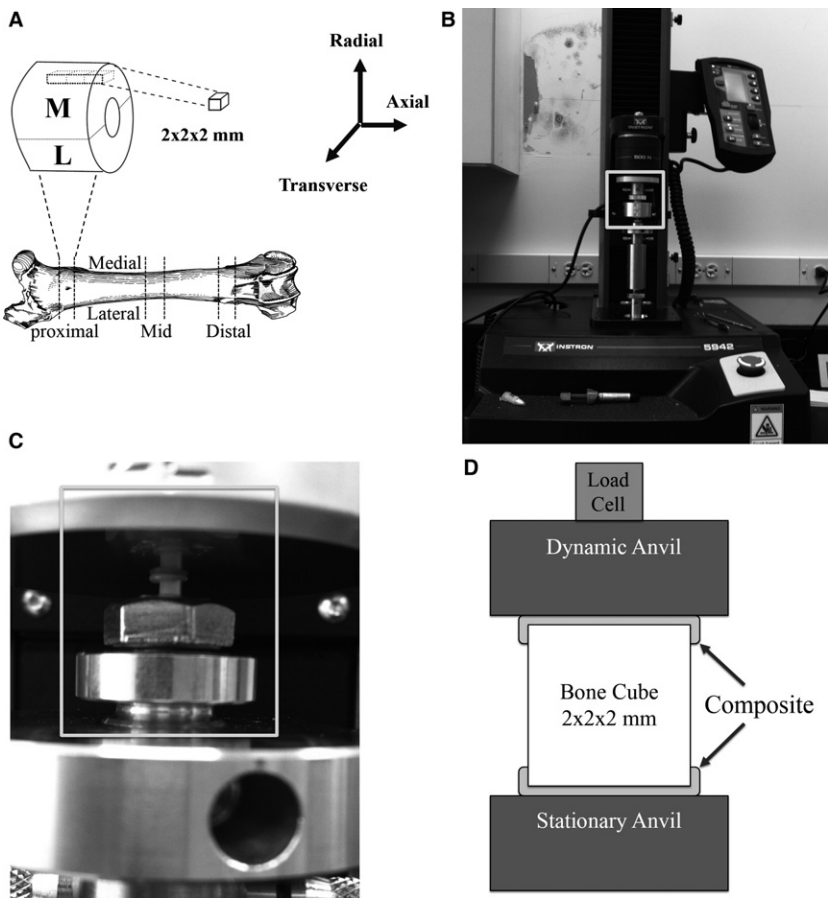

Fig. 1 (A) A schematic illustration of the deer femora and the location from which the cubes were cut. The cubes (measured 2 × 2 × 2 mm) were prepared from the femur proximomedial region, parallel to the three primary axes of the bone. (B) The universal testing machine (Instron 5942) in compression mode, used in the experiment. The white square frame marks the higher magnification inset shown in the next plate. (C) A blowup showing a bone cube loaded between the two anvils. A composite layer can be seen between the upper and lower cube surfaces and the upper and lower anvils, respectively. The white square frame marks the schematic inset shown in the next plate. (D) A schematic drawing of a mounted cube between the lower stationary anvil and the upper mobile anvil. A thin coat of composite was applied onto the cube's two loading surfaces to eliminate any possible stress concentration caused by small deviation from perfect parallel surfaces.

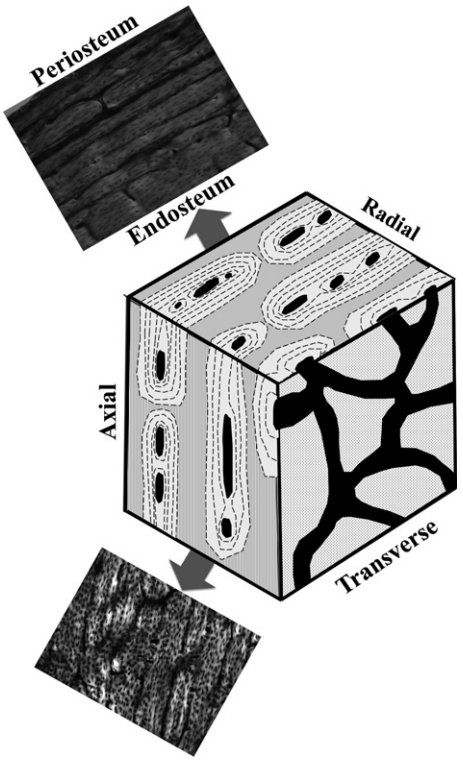

Fig. 2 A three-dimensional schematic drawing showing the structure of fibrolamellar bone (an orthotropic structure). Dark gray represents the parallel-fibered or woven bone scaffolding which is laid first, light gray represents the concentric lamellae which constitute the primary osteons, and black represents the primary osteons' central canals and Volkmann's canals. The two associated microscopic images are representative examples from our samples' histological slides, demonstrating and verifying the fibrolamellar bone structure of our bone cubes (upper image displays a transverse section and lower image displays a sagittal section).

Sample mounting

An Instron 5942 Single Column Table frame in compression mode was used as the universal testing machine in our study. Prior to mounting the first sample, the Instron machine was tested with an infinitely stiff sample (basically, pressing the two anvils against each other) to measure machine compliance. This compliance test was run with the exact same protocol as the actual cortical bone cube testing. The results of this calibration were fed back to the software (BLUEHILL 3.0®, Instron USA) to compensate for machine compliance (mostly, deformation of the load cell).

Twenty-four hours before each bone cube was tested, it was transferred from −20 to 4 °C and was allowed to thaw. To eliminate any possible stress concentration or non-pure compression due to slightly unparallel faces, a thin layer of dental composite material (Z250, 3M ESPE, St. Paul, MN, USA) was placed between the tested bone cube and the anvils of the Instron machine (Fig. 1C,D). The Z250 3M composite material was used due to its stiffness value (around 11GPa; '3M, Filtek Z250 - Universal Restorative System,' 1998), which is within the range of cortical bone stiffness values (Shahar et al. 2007). First, a thin layer of composite was placed over the lower, stationary anvil and the cube was gently pushed down onto the composite such that it was mounted in the appropriate orientation (i.e. axial, radial or transverse). Next, a second thin layer of composite was placed on the surface of the upper dynamic anvil and the anvil was lowered until contact with the cube (Fig. 1D). A more detailed explanation of the mounting sequence can be found in Shahar et al. (2007). Finally, the Z250 composite was polymerized for 60 s using a hand-held light-curing device (GadgetWorkz Woodpecker 5 W LED curing light) and water was added around the sample to keep it hydrated, simulating physiological conditions.

Mechanical compression testing

A small compression preload of about 5 N was applied at the beginning of each experiment, and only then were load and deformation data collected (measurements were taken every 0.1 s). Each cube was loaded at a rate of 50 μm min^{-1} until a load of 140 N was reached. Preliminary experiments which were run up to 400 N with several repetitions showed no evidence of damage and verified that the cubes were being tested within their elastic region. When the load reached 140 N the experiment ended and the upper dynamic anvil moved back to its original position (i.e. load below 5 N). This sequence was repeated nine times per mounting. The first six cycles served as mechanical conditioning of the viscoelastic sample till it achieved a steady state (Linde and Hvid, 1999) and the last three cycles were used to record the sample's stiffness in the measured orientation. After all nine loading cycles ended, the upper dynamic anvil was lifted, and the bone cube was dismounted and then remounted in a different orthogonal orientation. Thus, we were able to collect data from three repeated experiments of force-deformation measurements for each of the three orthogonal sample axes for each cube. After each cube was tested in all three orthogonal orientations, it was re-stored on water-saturated cloth at −20 °C.

After all 30 cubes were tested, we started the second phase of the study. Each cube was thawed again and maintained in water at 4 °C for 7 days. Next, the exact same protocol was repeated and bone cube stiffness was measured for the second time. Thus each cube was loaded separately twice in each orientation – once after 24 h of thawing and the second time after 7 days of thawing.

Data analysis

Stress values were calculated as the ratio between the force measurements and the cross-sectional area of the sample. Strain values were calculated as the ratio between the corrected movement of the anvil (corrected for machine compliance) and the original gap between the two anvils ('specimen height'). A stress vs. strain curve was created for each compression cycle and the Young's modulus determined from the 20–100 N linear region of the curve. For each bone cube in each orientation the three Young's moduli were averaged to give a final Young's modulus value.

To determine whether there was a significant difference in cortical bone Young's modulus between day zero and after 7 days at 4 °C, a one-tailed paired *t*-test was performed. Values smaller than 0.05 ($P < 0.05$) were considered to be statistically significant.

Results

Bone structure

Histological sections just above, below and to the sides of the tested cubes confirmed that the cortical bone structure

consists solely of fibrolamellar bone (Fig. 2). The transverse sections reveal primary osteons surrounded by parallel-fibered or woven bone, where newer bone is being laid closer to the periosteal side (Fig. 2, larger spaces appear closer to the periosteum, upper side of the transverse section).

Young's moduli at day 0 and day 7

As expected for fibrolamellar bone, the cortical bone demonstrated the greatest stiffness in the axial orientation, which is the physiological direction of loading (21.6 $\pm$ 3.3 GPa), followed by the transverse orientation (17.6 $\pm$ 3.0 GPa) and the radial orientation (14.9 $\pm$ 1.9 GPa) (Table 2, Fig. 3). All three orientations were statistically significantly different from each other ($P \ll 0.01$). After maintaining the cortical bone samples for 7 days at 4 °C, they showed a small non-significant decrease in stiffness (21.5 $\pm$ 3.5, 16.8 $\pm$ 3.1 and 14.8 $\pm$ 1.9 GPa for the axial, transverse and radial orientations, respectively; $P > 0.5$) (Table 2, Fig. 3).

Young's moduli variance

The dispersion of Young's moduli in each orientation, expressed as standard deviation (SD), is given in Table 2. Similar to the actual Young's moduli, the highest SD is observed in the axial direction, followed by the transverse and radial directions (3.3, 3.0 and 1.9 Gpa, respectively). This trend did not change after 7 days in 4 °C, although SD values tend to be larger at day 7 (3.5, 3.1 and 1.9 GPa in the axial, radial and transverse directions, respectively).

Discussion

The main goal of this study was to accurately determine the stiffness (Young's modulus) of fibrolamellar bone in compression in all three orthogonal directions. Our study shows that fibrolamellar bone stiffness clearly demonstrates an orthotropic behavior in which the greatest stiffness is in the axial direction (direction of loading) followed by the

Table 2 Young's moduli for all 30 cubes (SD in parentheses) in the axial (A), radial (R) and transverse (T) directions, 24 h after thawing the samples (0 days) and after storing the samples for additional 7 days in 4 °C (7 days). The last raw gives the average Young's modulus of each orientation with its SD (both actual value and as a percentage of the Young's modulus value).

		t = 0 days			t = 7 days		
Bone no.	Cube no.	A	R	T	A	R	T
1	1	22.49 (0.64)	14.39 (0.34)	15.91 (0.33)	22.99 (0.21)	17.02 (0.26)	14.53 (0.35)
1	2	22.56 (0.44)	14.98 (0.10)	13.46 (0.48)	23.44 (0.85)	13.46 (0.58)	13.71 (0.46)
1	3	27.40 (0.92)	12.23 (0.11)	18.85 (0.76)	17.92 (0.53)	14.30 (0.35)	14.25 (0.24)
1	4	25.59 (0.77)	14.92 (0.15)	13.16 (0.50)	20.53 (0.94)	13.58 (0.25)	13.25 (0.11)
1	5	19.89 (0.48)	13.73 (0.32)	12.97 (0.46)	24.21 (1.23)	16.54 (0.34)	12.63 (0.11)
1	6	20.02 (0.32)	13.09 (0.18)	15.09 (0.29)	16.45 (0.80)	12.75 (0.27)	13.90 (0.22)
1	7	25.69 (0.23)	15.71 (0.21)	18.78 (0.22)	16.76 (0.82)	13.66 (0.66)	15.31 (0.72)
2	1	22.31 (0.73)	13.99 (0.26)	14.38 (0.57)	20.84 (1.08)	14.28 (0.16)	18.64 (0.81)
2	2	20.06 (0.42)	16.44 (0.62)	14.73 (0.41)	19.70 (0.23)	13.28 (0.29)	19.90 (0.50)
2	3	23.79 (0.94)	13.81 (0.21)	16.56 (0.53)	20.85 (0.67)	16.57 (0.52)	18.35 (0.61)
2	4	21.90 (0.3)	15.20 (0.14)	19.45 (0.65)	16.73 (0.85)	11.58 (0.49)	13.43 (0.67)
2	5	19.92 (0.31)	14.25 (0.21)	21.54 (0.28)	23.06 (1.31)	15.09 (0.35)	18.49 (0.13)
2	6	18.05 (0.20)	12.88 (0.18)	20.08 (0.15)	28.59 (0.78)	14.51 (0.39)	18.75 (0.69)
2	7	16.71 (0.84)	16.08 (0.49)	20.59 (0.37)	20.89 (0.70)	11.72 (0.33)	19.23 (0.94)
2	8	20.76 (0.62)	12.41 (0.18)	18.76 (0.40)	17.29 (0.79)	14.63 (0.37)	15.29 (0.48)
3	1	21.10 (0.57)	16.66 (0.14)	19.03 (0.11)	20.86 (0.64)	13.63 (0.30)	13.22 (0.46)
3	2	20.07 (0.39)	16.58 (0.07)	14.69 (0.21)	19.68 (0.57)	17.30 (0.26)	15.71 (0.30)
3	3	21.02 (0.20)	14.69 (0.39)	19.01 (0.61)	21.74 (1.12)	17.48 (0.54)	17.97 (0.38)
3	4	28.36 (0.94)	16.59 (0.29)	18.70 (0.33)	19.53 (0.83)	15.79 (0.34)	16.79 (0.56)
3	5	24.51 (0.65)	17.48 (0.28)	19.04 (0.61)	24.17 (0.43)	16.24 (0.41)	19.27 (0.90)
4	1	25.83 (0.79)	21.39 (1.18)	26.07 (0.39)	28.76 (1.83)	16.70 (0.22)	17.86 (1.22)
4	2	23.76 (0.60)	15.96 (0.31)	22.60 (0.57)	26.12 (0.93)	19.22 (0.39)	27.14 (1.37)
4	3	20.36 (1.07)	15.46 (0.27)	17.75 (0.67)	19.48 (0.78)	14.88 (0.40)	14.33 (0.50)
4	4	20.47 (0.43)	15.24 (0.28)	19.03 (0.82)	21.76 (0.69)	16.65 (0.44)	20.30 (0.10)
4	5	32.57 (1.25)	15.64 (0.19)	19.00 (0.68)	25.08 (1.06)	14.35 (0.40)	19.75 (0.88)
5	1	23.01 (0.27)	13.88 (0.23)	17.83 (0.29)	24.91 (0.26)	13.33 (0.12)	15.78 (0.48)
5	2	25.98 (1.11)	14.78 (0.14)	17.10 (0.48)	22.39 (1.33)	13.69 (0.49)	18.41 (0.46)
5	3	21.18 (0.63)	12.75 (0.07)	14.95 (0.36)	15.54 (0.57)	12.76 (0.15)	14.37 (0.55)
5	4	21.64 (0.74)	12.29 (0.28)	15.67 (0.30)	19.87 (0.67)	12.29 (0.13)	13.84 (0.55)
5	5	21.32 (0.76)	12.99 (0.40)	14.19 (0.25)	25.83 (1.16)	15.45 (0.19)	19.47 (0.27)
	$\bar{X}$	21.61 (3.30)	14.88 (1.90)	17.63 (3.02)	21.53 (3.46)	14.76 (1.87)	16.80 (3.14)

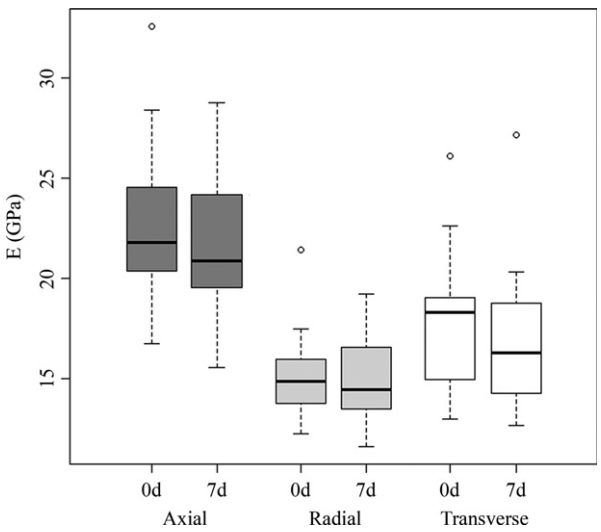

Fig. 3 A boxplot diagram showing the distribution of sample's Young's moduli in the three orthogonal orientations (axial in dark grey, radial in light grey and transverse in white) 24 h after thawing the samples (0 days) and after storing the samples for 7 days in 4 °C (7 days). The horizontal line inside the boxes is the median. Box hinges represent 25th and 75th percentiles. Whiskers represent minimum and maximum measured values which are not statistical outliers. An outlier is defined as a data point that is located 1.5 times interquartile range above the upper quartile and bellow the lower quartile; outliers are represented by a circle sign (These outliers appear as such only in the figure, no values were excluded from the statistical calculations).

transverse and radial directions (21.6 ± 3.3, 17.6 ± 3.0 and 14.9 ± 1.9 Gpa, respectively). A second goal was to determine whether the stiffness of fibrolamellar bone would decrease after bone samples were kept thawed for 7 days at 4 °C. Our results indicate that this treatment did not affect bone stiffness significantly ($P > 0.05$) and that bone Young's moduli decreased on average by less than 1% in the axial and radial directions and by less than 5% in the transverse direction (Table 2, Fig. 3).

To the best of our knowledge, our study is the first to present extensive Young's moduli for fibrolamellar bone in all three orthogonal directions, based on a large sample size ($n = 30$) in compression (reproducing the physiological *in vivo* loading of the proximomedial femur). Only a few other studies report the stiffness (Young's modulus) of fibrolamellar bone (Table 1) and most of them used a relatively small sample size ($n = 4$–16). Furthermore, the majority of these studies tested given samples only in one orientation, mostly axial (Table 1). Only one other study, Van Buskirk & Ashman (1981), tested 20 samples and measured their stiffness in all three orthogonal directions; however, that study measured bone stiffness using an acoustic technique (ultrasound). Since ultrasound measurements are indirect, calculating stiffness from the velocity of wave propagation and tissue density (measured or assumed), it is important to validate these results with a more 'direct' technique.

The only previous study which tested fibrolamellar bone Young's modulus in compression was Reilly et al. (1974). They tested 10 samples from two bovine femora only in the axial direction and found an average Young's modulus of 30.3 Gpa, which is around 30% stiffer than our results. Conversely, the majority of the other previous studies (Table 1) found axial stiffness values that were lower than those reported by Reilly and his colleagues. For example, the two other studies with relatively large sample sizes, 20 samples (Van Buskirk & Ashman, 1981) and 35 samples (Martin & Boardman, 1993) found axial Young's modulus results very similar to our own (21.9 and 21.0 Gpa, respectively, Table 1).

There are several key differences between Reilly et al. (1974) and our experiment. First, we were testing deer femora, whereas Reilly et al. tested bovine femora. Despite the fact that both species share the same bone tissue type (i.e. fibrolamellar bone), some structural and mechanical variances may exist. Mori et al. (2003) found that young foals had a different variant of fibrolamellar bone tissue compared with young calves. Secondly, whereas Reilly et al. used samples from the mid-diaphysis, we harvested our samples from the proximal diaphysis. As bone diameter is the smallest at mid-diaphysis, bone cross-sectional area is most likely also smaller, and thus the stresses in the tissue are higher. One way to overcome these higher stresses is by increasing mineralization, which will also increase bone stiffness. Indeed, in a recent experiment done in our lab we harvested similar cube samples ($n = 12$) from the same five femora, this time from the mid-diaphysis. The average Young's modulus value was 30.87 ± 5.0 GPa, almost identical to the results found by Reilly and his colleagues. Finally, whereas we used cubical samples, the samples Reilly et al. used were dumbbell-shaped, which they described as 'suitable for both compression and tension'. A dumbbell-shaped sample is very unique and uncommon in compression testing and it is uncertain how this method of testing affected their results.

There are only two previous studies which address the stiffness of fibrolamellar bone in the transverse and radial directions (this excludes the Reilly & Burstein, 1975, study, which presents only one Young's modulus value perpendicular to the long axis). Interestingly enough, in both orthogonal directions our results fit exactly in between these two previously reported values. In the transverse direction our Young's modulus value of 17.6 GPa falls in between the Van Buskirk & Ashman (1981) value of 14.6 GPa and the Lipson & Katz (1984) value of 21.4 GPa. Similarly, in the radial direction, our Young's modulus value of 14.9 GPa falls in between the Van Buskirk & Ashman (1981) value of 11.6 GPa and the Lipson & Katz (1984) value of 17.3 GPa. Again, there may be several reasons for these differences. First, and similar to Reilly et al. (1974), these two studies used

bovine femora whereas we tested deer femora. Secondly, both these studies used ultrasound to measure their stiffness values. As discussed above, this technique is an indirect way to measure bone stiffness and it may be heavily affected by an inaccurate measurement or an estimation of bone density. Regardless of these relatively small differences, it is important to emphasize that all three studies found that fibrolamellar bone demonstrates orthotropic mechanical behavior in which the axial direction was found to be the stiffest, following by the transverse and radial directions, respectively.

Our second goal was to test the stiffness of fibrolamellar bone after bone samples were kept thawed for 7 days at 4 °C. As there is very little elaboration in some papers on how much time they allowed their samples to thaw before testing them, we thought it would be interesting to take this parameter to the 'extreme' and see the effect of leaving the samples for 7 days in water at 4 °C before testing them again. In addition, since at these temperatures lytic enzymes are still partly active, this question has clinical relevance when bone samples are being stored before they are being transplanted to a patient (Tomford et al. 1983). As we tested our bone cubes within their elastic region we were able to reuse them again after 7 days and compare each sample, in each direction, to determine whether the stiffness of our bones deteriorated significantly. Previously, Tennyson et al. (1972) tested the axial stiffness of Haversian bone from 2-year-old bovine femora under impact loading (Split-Hopkinson-bar technique). Similar to our results, they did not find a significant decrease in Young's modulus up to 1 week of storage in water at a temperature of 1.7 °C. However, they reported a decrease of about 33% in bone stiffness during the second week which stayed constant thereafter till the end of the experiment (38 days). Other studies explored the effect of storage time on bone tissue stiffness and strength in samples kept at room temperature. Ashman et al. (1982) tested the axial Young's modulus of eight canine cortical bone specimens kept in water at room temperature for 100 h (about 4 days). They found a consistently slow decrease in stiffness during that time, yet the reduction in stiffness never exceeded 2% (no statistical test was reported). Kääb et al. (1998) investigated the effect of keeping human vertebral cancellous bone samples at room temperature for 3.5 days before performing screw pullout tests and measuring pullout strength. They found no significant difference in pullout strength between samples which were tested fresh and those tested up to 84 h later. These results indicate that regardless of bone tissue type (Haversian bone, fibrolamellar bone or trabecular bone) and regardless of temperature (as long as it is below normal body temperature), bone tissue retains its mechanical properties (with a possible slight non-significant decrease), at least for several days. Contrary to these findings, Linde & Sørensen (1993) found a decrease of 10% in the axial Young's modulus of trabecular bone samples from human proximal tibiae after storing them in saline for just 24 h at room temperature. However, it is worth mentioning that they tested the same trabecular samples twice. Although the researchers reported that the samples were loaded within their elastic region (i.e. non-destructive compression test) it is impossible to validate that no damage occurred to any of the trabeculae during their first test. Multiple previous studies have shown that some trabeculae may buckle, crack or even break very early into the compression test, much before the actual yield point is achieved. This is particularly true for samples taken from older, possibly osteoporotic, individuals (the samples Linde & Sorensen obtained were from two individuals, one of them 61 years old).

Another valuable outcome derived from our large sample size ($n = 30$) and the fact we were testing each sample in all three orthogonal directions was our ability to evaluate our data variability. When we compare the standard deviation (SD) of our results in the axial, transverse and radial directions it is clear that the variability of our data is significantly larger in the axial direction than in the radial direction (Table 2). In the axial direction the SD value is 3.3 Gpa, whereas in the radial direction it is only 1.9 GPa. In the transverse direction the SD value is between the axial and radial directions (3.0 Gpa). Raichlen et al. (2015) revealed a correlation between trabecular anisotropy SD values and kinematic variation and used it to demonstrate the decrease in kinematic pattern variability of young children as they become older, more balanced and stabilize their stride. Similarly, we suggest that the difference between our measured SD values reflects the actual functional history of the bone (i.e. bone adaptation, also referred to as 'Wolff's law'). As appendicular bones (e.g. femur) are loaded primarily in the axial direction and as different individuals differ in their weight and amount of activity, we would expect the highest variability in mechanical properties to be in that direction. Using the same logic, the radial direction which is loaded the least frequently (bone is rarely loaded directly in the axial and transverse directions) should demonstrate the least variability among different individuals. Despite the fact that bone adaptation ('Wolff's law') refers to both cortical and trabecular bone tissues, it is much easier to demonstrate its effects in trabecular bone, which is a 3D porous structure. Consequently, most studies demonstrated bone adaptation in trabecular bone tissue (Biewener et al. 1996; Pontzer et al. 2006; Barak et al. 2011, 2013) and only a handful of studies discuss this phenomenon in cortical bone (Pearson & Lieberman, 2004; Shaw & Ryan, 2012). Our results supplement these previous studies and support the prediction that bone adaptation to loading takes affect also in cortical bone tissue.

In conclusion, this study investigated the stiffness of fibrolamellar bone. Compared with previous studies our study has several advantages: a large sample size, each sample was tested in all three orthogonal directions, and a testing technique (compression) that simulated the physiological

way the bone is being loaded *in vivo*. Our study demonstrated that fibrolamellar bone is an orthotropic structure and that it is stiffest in the axial direction, followed by the transverse and radial directions. In addition, our results showed that keeping fibrolamellar bone in water for 7 days at 4 °C did not decrease its stiffness in any of the three orthogonal directions. Finally, we suggest that the relatively high variance of stiffness values in the axial direction compared with the radial direction is correlated with the variability of bone loading between individuals in the axial and radial directions, thus supporting 'Wolff's law' of bone adaptation.

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Contributors

John W. Barrera helped to design the study, did the experimental work and helped to write the paper. Adeline Le Cabec contributed to data analysis and interpretation and helped to write the paper. Meir M. Barak designed the study, helped with the experimental work and wrote the paper. All authors revised the paper critically for intellectual content and approved the final version. All authors agree to be accountable for the work and to ensure that any questions relating to the accuracy and integrity of the paper are investigated and properly resolved.

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